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(19) Europäisches Patentamt
European Patent Office
Office européen des brevets

(11) EP 1 088 891 A1

(12) **EUROPEAN PATENT APPLICATION**

(43) Date of publication:
04.04.2001 Bulletin 2001/14

(21) Application number: **99119268.3**

(22) Date of filing: **28.09.1999**

(51) Int Cl.[7]: **C12N 15/55**, C12N 15/54, C12N 9/22, C12N 9/12, C12Q 1/68, C12P 19/34

(84) Designated Contracting States:
AT BE CH CY DE DK ES FI FR GB GR IE IT LI LU MC NL PT SE
Designated Extension States:
AL LT LV MK RO SI

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Remarks:
The applicant has subsequently filed a sequence listing and declared, that it includes no new matter.

(54) **Thermostable enzyme promoting the fidelity of thermostable DNA polymerases - for improvement of nucleic acid synthesis and amplification in vitro**

(57) A purified thermostable enzyme is derived form the thermophilic archaebacterium *Archaeoglobus fulgidus*. The enzyme can be native or recombinant, is stable under PCR conditions and exhibits double strand specific exonuclease activity. It is a 3'-5'exonuclease and cleaves to produce 5'-mononucleotides. Thermostable exonucleases are useful in many recombinant DNA techniques, in combination with a thermostable DNA polymerase like *Taq* especially for nucleic acid amplification by the polymerase chain reaction (PCR).

Figure 6A

Error rates of different DNA polymerases in PCR

Polymerase	Template conc. (ng)	yield (ng)	DNA duplications d	blue colonies lacI⁻	white colonies lac⁻	total number of colonies	% lac⁻	error rate (f_{349})
Taq Ch.	10	11650	10.2	130	2261	2391	5.4	1.57×10^{-3}
HiFi Ch.	10	11550	10.2	40	5458	5498	0.72	2.06×10^{-4}
Pwo	10	9675	9.9	17	5891	5908	0.29	8.32×10^{-5}
Taq/Exo 1	10	11550	10.2	94	4291	4385	2.14	6.10×10^{-4}
Taq/Exo 2	10	11125	10.1	146	7644	7790	1.87	5.36×10^{-4}
Taq/Exo 3	10	8500	9.7	133	8188	8321	1.6	4.74×10^{-4}
Taq/Exo 4	10	1292	7	79	7236	7315	1.08	4.44×10^{-4}
Taq/Exo 5	10	236	4.6	25	2674	2724	0.92	1.16×10^{-3}*

* Due to the unfavorable ratio of Taq:Exo the product yield was low. This results in an apparently low amplification efficiency d, which is an important parameter in the formula used for the calculation of the error rate.

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Description

FIELD OF THE INVENTION

[0001] The present invention is related to the field of molecular biology, and more particular, to polynucleotide synthesis. The present invention also relates to a substantially pure thermostable exonuclease, the cloning and expression of a thermostable exonuclease III in *E.coli,* and its use in amplification reactions. The invention facilitates the high fidelity amplification of DNA under conditions which allow decontamination from carry over and the synthesis of long products. The invention may be used for a variety of industrial, medical and forensic purposes.

BACKGROUND OF THE INVENTION

[0002] *In vitro* nucleic acid synthesis is routinely performed with DNA polymerases with or without additional polypeptides. DNA polymerases are a family of enzymes involved in DNA replication and repair. Extensive research has been conducted on the isolation of DNA polymerases from mesophilic microorganisms such as *E.coli.* See, for example, Bessman et al. (1957) *J. Biol. Chem.* **223**:171-177, and Buttin and Kornberg, (1966)*J. Biol. Chem.* **241**:5419-5427.

[0003] Research has also been conducted on the isolation and purification of DNA polymerases from thermophiles, such as *Thermus aquaticus.* Chien, A., (1976) et al. *J. Bacteriol.* **127**:1550-1557 discloses the isolation and purification of a DNA polymerase with a temperature optimum of 80°C from *Thermus aquaticus* YT1 strain. United States Patent No. 4,889,818 discloses a purified thermostable DNA polymerase from *T. aquaticus,* Taq polyrnerase, having a molecular weight of about 86,000 to 90,000 daltons. In addition, European Patent Application 0 258 017 discloses Taq polymerase as the preferred enzyme for use in the PCR process.

[0004] Research has indicated that while Taq DNA polymerase has a 5'-3' polymerase-dependent exonuclease function, *Taq* DNA polymerase does not possess a 3'-5' *exonuclease III* function (Lawyer, F.C. et al., (1989) *J. Biol. Chem.,* **264**:6427-6437; Bernad A., et al. (1989) *Cell***59**:219). The 3'-5' *exonuclease III* activity of DNA polymerases is commonly referred to as „proofreading activity". The 3'-5' *exonuclease III* activity removes bases which are mismatched at the 3' end of a primer-template duplex. The presence of 3'-5' exonuclease activity may be advantageous as it leads to an increase in fidelity of replication of nucleic acid strands and to the elongation of prematurely terminated products . As *Taq* DNA polymerase is not able to remove mismatched primer ends it is prone to base incorporation errors, making its use in certain applications undesirable. For example, attempting to clone an amplified gene is problematic since any one copy of the gene may contain an error due to a random misincorporation events. Depending on the cycle in which that error occurs (e.g., in an early replication cycle), the entire DNA amplified could contain the erroneously incorporated base, thus, giving rise to a mutated gene product.

[0005] There are several thermostable DNA polymerases known in the art which exhibit 3'- 5'exonuclease activity, like B-type polymerases from thermophilic Archaebacteria which are used for high fidelity DNA amplification. Thermostable polymerases exhibiting 3'- 5'exonuclease activity may be isolated or cloned from *Pyrococcus* (Purified thermostable *Pyrococcus furiosus* DNA polymerase, Mathur E., Stratagene, WO 92/09689, US 5,545,552; Purified thermostable DNA polymerase from *Pyrococcus species,* Comb D. G. et al., New England Biolabs, Inc., EP 0 547 359; Organization and nucleotide sequence of the DNA polymerase gene from the archaeon *Pyrococcus furiosus,* Uemori T. et al. (1993) *Nucl. Acids Res.*, **21**:259-265.), from *Pyrodictium spec.* (Thermostable nucleic acid polymerase, Gelfand D. H., F. Hoffmann-La Roche AG, EP 0 624 641; Purified thermostable nucleic acid polymerase and DNA coding sequences from *Pyrodictium species,* Gelfand D. H., Hoffmann-La Roche Inc., US 5,491,086), from *Thermococcus* (e. g. Thermostable DNA polymerase from *Thermococcus spec.* TY, Niehaus F., et al. WO 97/35988; Purified *Thermocccus barossii* DNA polymerase, Luhm R. A., Pharmacia Biotech, Inc., WO 96/22389; DNA polymerase from *Thermococcus barossii* with intermediate exonuclease activity and better long term stability at high temperature, useful for DNA sequencing, PCR etc., Dhennezel O.B., Pharmacia Biotech Inc., WO 96/22389; A purified thermostable DNA polymerase from *Thermococcus litoralis* for use in DNA manipulations, Comb D. G., New England Biolabs, Inc., US 5,322,785, EP 0 455 430; Recombinant thermostable DNA polymerase from Archaebacteria, Comb D. G., New England Biolabs, Inc., US 5,352,778, EP 0 547 920, EP 0 701 000; New isolated thermostable DNA polymerase obtained from *Thermococcus gorgonarius,* Angerer B. et al. Boehringer Mannheim GmbH, WO 98/14590.

[0006] Another possibility of conferring PCR in the presence of a proofreading function is the use of a mixture of polymerase enzymes, one polymerase exhibiting such a proofreading activity. (e.g. Thermostable DNA polymerase with enhanced thermostability and enhanced length and efficiency of primer extension, Barnes W. M., US 5,436,149, EP 0 693 078; Novel polymerase compositions and uses thereof, Sorge J. A., Stratagene, WO 95/16028). It is common practice to use a formulation of a thermostable DNA polymerase comprising a majority component of at least one thermostable DNA polymerase which lacks 3'-5' exonuclease activity and a minority component exhibiting 3'- 5' exonuclease activity e.g. *Taq* polymerase and *Pfu* DNA polymerase. In these mixtures the processivity is conferred by the pol I-type enzyme like *Taq* polymerase, the proofreading function by the thermostable B-type polymerase like *Pfu.* High

fidelity DNA synthesis is one desirable parameter in nucleic acid amplification, another important feature is the possibility of decontamination.

[0007] The polymerase chain reaction can amplify a single molecule over a billionfold. Thus, even minuscule amounts of a contaminant can be amplified and lead to a false positive result. Such contaminants are often poducts from previous PCR amplifications (carry-over contamination). Therefore, researchers have developed methods to avoid such a contamination.

[0008] The procedure relies on substituting dUTP for TTP during PCR amplification to produce uracil-containing DNA (U-DNA). Treating subsequent PCR reaction mixtures with Uracil-DNA-Glycosylase (UNG) prior to PCR amplification the contaminating nucleic acid is degraded and not suitable for amplification. dUTP can be readily incorporated by poll-type thermostable polymerases but not B-type polymerases (G. Slupphaug, et al. (1993) *Anal. Biochem.* **211**: 164-169) Low incorporation of dUTP by B-type polymerases limits their use in laboratories where the same type of template is repeatedly analyzed by PCR amplification.

[0009] Thermostable DNA polymerases exhibiting 3' - 5'exonuclease activity were also isolated from eubacterial strains like *Thermotoga* (Thermophilic DNA polymerases from *Thermotoga neapolitana,* Slater M. R. et al. Promega Corporation, WO 96/41014; Cloned DNA polymerases from *Thermotoga neapolitana* and mutants thereof, Hughes A. J. et al., Life Technologies, Inc. WO 96/10640; Purified thermostable nucleic acid polymerase enzyme from *Termotoga maritima,* Gelfand D. H. et al., CETUS Corporation, WO 92/03556) These enzymes have a strong 3'-5'exonuclease activity which is able to eliminate misincorporated or mismatched bases. A genetically engineered version of this enzyme is commercially available as ULTma, a DNA polymerase which can be used without additional polypeptides for the PCR process. This enzyme is able to remove misincorporated bases, incorporate dUTP, but the fidelity is for unknown reasons not higher than that of Taq polymerase (Accuracy of replication in the polymerase chain reaction. Diaz R. S. et al. *Braz. J. Med. Biol. Res.* (1998) **31**: 1239-1242; PCR fidelity of *Pfu* DNA polymerase and other thermostable DNA polymerases, Cline J. et al., *Nucleic Acids Res.* (1996) **24**:3546-3551).

[0010] For high fidelity DNA synthesis another alternative to the use of B-type polymerases or mixtures containing them is the use of thermophilic DNA polymerase III holoenzyme, a complex of 18 polypeptide chains. These complexes are identical to the bacterial chromosomal replicases, comprising all the factors necessary to synthesize a DNA strand of several hundred kilobases or whole chromosomes. The 10 different subunits of this enzyme, some of which are present in multiple copies, can be produced by recombinant techniques, reconstituted and used for *in vitro* DNA synthesis. As a possible use of these complexes PCR amplification of nucleic acis of several thousand to hundreds of thousand base pairs is proposed. (Enzyme derived from thermophilic organisms that functions as a chromosomal replicase, and preparation and uses thereof, Yurieva O.et al., The Rockefeller University, WO 98/45452; Novel thermophilic polymerase III holoenzyme, McHenry C., ENZYCO Inc., WO 99/13060)

DESCRIPTION OF THE INVENTION

[0011] It was aimed according to this invention to develop a high fidelity PCR system which is preferably concomitantly able to incorporate dUTP. According to the present invention a thermostable enzyme exhibiting 3' exonuclease-activity but no polymerase activity is provided whereas this enzyme enhances fidelity of an amplification process when added to a second enzyme exhibiting polymerase activity. The enzyme provided can excise mismatched primer ends to allow the second enzym exhibiting polymerase activity as e.g. Taq polymerase to reassociate and to reassume elongation during a process of synthezising DNA. The inventive enzyme is able to cooperate as proofreading enzyme with a second enzyme exhibiting polymerase activity The enzyme that was found to be appropriate for this task is e.g. a thermostable *exonuclease III.* Preferred is an *exonuclease III* working from the 3' to 5' direction, cleaving 5' of the phosphate leaving 3' hydroxyl groups and ideally working on double stranded DNA only. The 3'- 5'exonuclease functions of DNA polymerases are active on double and single stranded DNA. The latter activity may lead to primer degradation, which is undesired in PCR assays . It is preferred that the enzyme is active at 70 °C to 80 °C, stable enough to survive the denaturation cycles and inactive at lower temperatures to leave the PCR products undegraded after completion of the PCR process. Enzymes exhibiting these features can be derived from thermophilic eubacteria or related enzymes from thermophilic archaea. Genomes of three thermostable archaebacteria are sequenced, *Methanococcus jannaschii* (Complete Genome Sequence of the Methanogenic Archaeon, *Methanococcus jannaschii,* Bult C.J. et al., (1996) *Science* **273**:1058-lo72), *Methanobacterium thermoautotrophicum* (Complete genomic sequence of *Methanobacterium thermoautotrophicum* ΔH: Functional Analysis and Comparative Genomics, Smith D.R. et al., J. *of Bacteriology* (1997) **179**: 7135-7155) and *Archaeoglobus fulgidus* (The complete genome sequence of the hyperthermophilic, sulfate-reducing archaeon *Archaeoglobus fulgidus,* Klenk H.-P. et al. (1997) *Nature* **390**: 364-370).

[0012] In particular, there is provided a thermostable enzyme obtainable from *Archaeoglobus fulgidus,* which catalyzes the degradation of mismatched end of primers or polynucleotides in the 3' to 5' direction in double stranded DNA. The gene encoding the thermostable exonuclease III obtainable from *Archaeoglobus fulgidus* (Afu) was cloned, expressed in *E.coli* and isolated. The enzyme is active under the incubation and temperature conditions used in PCR

reactions. The enzyme supports DNA polymerases like *Taq* in performing DNA synthesis at low error rates and synthesis of products of more than 3 kb on genomic DNA - the upper range of products synthesized by *Taq* polymerase - in good yields with or without dUTP present in the reaction mixture. Preferably, 50-500 ng of the exonuclease III obtainable from Afu were used per 2,5 U of Taq polymerase in order to have an optimal PCR performance. More preferably is the use of 67 ng-380 ng of the exonuclease III obtainable from Afu per 2,5 U of the Taq polymerase in the PCR reaction.

[0013] Thus, the inventive enzyme is able to cooperate as proofreading enzyme with *Taq* polymerase. The advantage of the use of the inventive enzyme in comparison to other enzymes is that the inventive enzyme is preferably active on double stranded DNA. The thermostable enzyme of this invention may be used for any purpose in which such enzyme activity is necessary or desired. In a particularly preferred embodiment the enzyme is used in combination with a thermostable DNA polymerase in the nucleic acid amplification reaction known as PCR in order to remove mismatched primer ends which lead to premature stops, to provide primer ends which are more effectively elongated by the polymerase, to correct for base incorporation errors and to enable the polymerase to produce long PCR products.

[0014] Further, subject of the present invention is a composition comprising a first thermostable enzyme exhibiting 3'-exonuclease-activity but no polymerase activity and a second enzyme exhibiting polymerase activity whereas the fidelity of an amplification process is enhanced by the use of this composition in comparison to the use of the second enzyme alone.In a preferred embodiment the second enzyme of the inventive composition is lacking proofreading activity. Mostly preferred, the second enzyme is Taq polymerase.

[0015] A further subject of the present invention is a method of DNA synthesis using a mixture comprising a first thermostable enzyme exhibiting 3'-exonuclease-activity but no polymerase activity and a second enzyme exhibiting polymerase activity. According to this method prematurely terminated chains are trimmed by degradation from 3' to 5'. Mismatched ends of either a primer or the growing strand are removed according to this method.

[0016] The invention further comprises a method according to the above description whereas dUTP is present in the reaction mixture, replacing TTP. It is preferred that according to this method UNG is used for degradation of contaminating nucleic acids.

[0017] Preferably, according to this method the mixture of a

- first thermostable enzyme exhibiting 3'-exonuclease-activity but no polymerase activity and
- a second enzyme exhibiting polymerase activity

produces PCR products with lower error rates compared to PCR products produced by the second enzyme exhibiting polymerase activity in absence of the first thermostable enzyme exhibiting 3'-exonuclease-activity but no polymerase activity. The method in which the mixture of first thermostable enzyme exhibiting 3'-exonuclease-activity but no polymerase activity and a second enzyme exhibiting polymerase activity produces PCR products of greater length compared to PCR products produced by the second enzyme exhibiting polymerase activity in absence of the first thermostable enzyme exhibiting 3'-exonuclease-activity but no polymerase activity. Further, the first thermostable enzyme exhibiting 3'-exonuclease-activity but no polymerase activity is related to the Exonuclease III of E. coli, but thermostable according to this method. A further embodiment of the above described method is the method whereas PCR products with blunt ends are obtained.

[0018] Subject of the present invention are also methods for obtaining the inventive thermostable enzyme exhibiting 3' exonuclease-activity but no polymerase acitvity and means and materials for producing this enzyme as e.g. vectors and host cells (e.g. host dell DSM no. 13021).

[0019] The following examples are offered for the purpose of illustrating, not limiting, the subject invention.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

Brief description of the drawings

[0020]

Figure 1:
DNA sequence and the deduced amino acid sequence of the gene encoding the DNA polymerase from exonuclease III of *Archaeoglobus fulgidus*.

Figure 2:
Resistance to heat denaturation of the recombinant exonuclease III of *Archaeoglobus fulgidus* expressed in *E.coli* as described in Example V.

Lane 1: Incubation at 50°C
Lane 2: Incubation at 60°C
Lane 3: Incubation at 70°C
Lane 4: Incubation at 80°C
Lane 5: Incubation at 90°C
Lane 6: *E.coli* host cell extract not transformed with gene encoding *Afu* exonuclease III
Lane 7: Exonuclease III of *E.coli*
Lane 8: Molecular weight marker

Figure 3:
Exonuclease activity of Afu exonuclease III on DNA fragments as described in Example VI.

Lane 1: 10 units *E.coli* exonuclease III, incubation at 37°C
Lane 2: 50 ng of *Afu* exonuclease III, incubation at 72°C
Lane 3: 100 ng of *Afu* exonuclease III, incubation at 72°C
Lane 4: 150 ng of *Afu* exonuclease III, incubation at 72°C
Lane 5: 100 ng of *Afu* exonuclease III, incubation at 72°C
Lane 6: 200 ng of *Afu* exonuclease III, incubation at 72°C
Lane 7: 300 ng of *Afu* exonuclease III, incubation at 72°C
Lane 8: 250 ng of *Afu* exonuclease III, incubation at 72°C
Lane 9: 750 ng of *Afu* exonuclease III, incubation at 72°C
Lane 10: 1 µg of *Afu* exonuclease III, incubation at 72°C
Lane 11: 500 ng of *Afu* exonuclease III, incubation at 72°C
Lane 12: 1 µg of *Afu* exonuclease III, incubation at 72°C
Lane 13: 1.5 µg of *Afu* exonuclease III, incubation at 72°C
Lane 14: 1.5 µg of *Afu* exonuclease III, incubation at 72°C
Lane 15: 3 µg of *Afu* exonuclease III, incubation at 72°C
Lane 16: 4.5 µg of *Afu* exonuclease III, incubation at 72°C
Lane 17: 7.6 µg of *Afu* exonuclease III, incubation at 72°C
Lane 18: 15.2 µg of *Afu* exonuclease III, incubation at 72°C
Lane 19: 22.8 µg of *Afu* exonuclease III, incubation at 72°C
Lane 20: no exonuclease added

Figure 4:
Principle of the mismatch correction assay.

Figure 5:
Mismatched primer correction in PCR as described in Example VII.

Lane 1: DNA Molecular Weight Marker V (ROCHE Molecular Biochemicals No. 821705)
Lane 2: G:A mismatched primer, amplification with *Taq* DNA polymerase
Lane 3: same as in lane 2, but subsequently cleaved with BsiEI
Lane 4: G:A mismatched primer, amplification with Expand HiFi PCR System
Lane 5: same as in lane 4, but subsequently cleaved with BsiEI
Lane 6: G:A mismatched primer, amplification with *Taq* polymerase/*Afu* exonuclease III
Lane 7: same as in lane 6, but subsequently cleaved with BsiEI
Lane 8: G:A mismatched primer, amplification with *Tgo* DNA polymerase
Lane 9: same as in lane 8, but subsequently cleaved with BsiEI
Lane 10: G:T mismatched primer, amplification with *Taq* DNA polymerase
Lane 11: same as in lane 10, but subsequently cleaved with BsiEI
Lane 12: G:T mismatched primer, amplification with Expand HiFi PCR System
Lane 13: same as in lane 12, but subsequently cleaved with BsiEI
Lane 14: G:T mismatched primer, amplification with *Taq* polymerase/*Afu* exonuclease III
Lane 15: same as in lane 14, but subsequently cleaved with BsiEI
Lane 16: G:T mismatched primer, amplification with *Tgo* DNA polymerase
Lane 17: same as in lane 16, but subsequently cleaved with BsiEI
Lane 18: DNA Molecular Weight Marker V
Lane 19: DNA Molecular Weight Marker V

Lane 20: G:C mismatched primer, amplification with *Taq* DNA polymerase
Lane 21: same as in lane 20, but subsequently cleaved with BsiEI
Lane 22: G:C mismatched primer, amplification with Expand HiFi PCR System
Lane23: same as in lane 22, but subsequently cleaved with BsiEI
Lane 24: G:C mismatched primer, amplification with *Taq* polymerase/*Afu* exonuclease III
Lane 25: same as in lane 24, but subsequently cleaved with BsiEI
Lane 26: G:C mismatched primer, amplification with *Tgo* DNA polymerase
Lane 27: same as in lane 26, but subsequently cleaved with BsiEI
Lane 28: CG:AT mismatched primer, *Taq* DNA polymerase
Lane 29: same as in lane 28, but subsequently cleaved with BsiEI
Lane 30: CG:AT mismatched primer, Expand HiFi PCR System
Lane 31: same as in lane 2, but subsequently cleaved with BsiEI
Lane32: CG:AT mismatched primer, *Taq* polymerase/*Afu* exonuclease III
Lane 33: same as in lane 2, but subsequently cleaved with BsiEILane 34: CG:AT mismatched primer, amplification with *Tgo* DNA polymerase
Lane 35: same as in lane 2, but subsequently cleaved with BsiEI
Lane 36: DNA Molecular Weight Marker V.

Figure 6A:
Error rates of different polymerases in PCR

Figure 6B:
Improvement of fidelity by *Afu* exonuclease III present in the PCR mixture as described in Example VIII.
The ratio of blue:white colonies were blottet and various mixtures of *Taq* DNA polymerase and *Afu* exonuclease III (Taq/Exo 1, Taq/Exo 2, Taq/Exo 3, Taq/Exo 4, Taq/Exo 5 corresponding to 2.5 units of *Taq* DNA polymerase mixed with 125 ng, 175 ng, 250 ng, 375 ng and 500 ng of *Afu* exonuclease III, respectively) were tested in compairison to *Taq* DNA polymerase (Taq), Expand HiFi PCR System (HiFi) and *Pwo* DNA polymerase (Pwo).

Figure 7:
Incorporation of dUTP by the *Taq* DNA polymerase/*Afu* exonuclease III mixture as described in Example IX.

Lane 1: DNA Molecular Weight Marker XIV (Roche Molecular Biochemicals No. 1721933)
Lane 2: Amplification with 2.5 units *Taq* DNA polymerase
Lane 3: Amplification with 2.5 units *Taq* DNA polymerase and 125 ng of *Afu* exonuclease III
Lane 4: Amplification with 2.5 units *Taq* DNA polymerase and 250 ng of *Afu* exonuclease III
Lane 5: Amplification with 2.5 units *Taq* DNA polymerase and 375 ng of *Afu* exonuclease III
Lane 6: Amplification with 2.5 units *Taq* DNA polymerase and 500 ng of *Afu* exonuclease III

Figure 8:
Degradation of dUTP containing PCR products by Uracil-DNA Glycosylase as described in Example IX.

Lane 1: DNA Molecular Weight Marker XIV (Roche Molecular Biochemicals No. 1721933)
Lane 2: 1 µl of the amplification product obtained with *Taq* DNA polymerase and 125 ng of *Afu* exonuclease III and subsequent UNG and heat treatment.
Lane 3: 2 µl of the amplification product obtained with *Taq* DNA polymerase and 125 ng of *Afu* exonuclease III and subsequent UNG and heat treatment.
Lane 4: 3 µl of the amplification product obtained with *Taq* DNA polymerase and 125 ng of *Afu* exonuclease III and subsequent UNG and heat treatment.
Lane 5: 4 µl of the amplification product obtained with *Taq* DNA polymerase and 125 ng of *Afu* exonuclease III and subsequent UNG and heat treatment. Lane 6: 5 µl of the amplification product obtained with *Taq* DNA polymerase and 125 ng of *Afu* exonuclease III and subsequent UNG and heat treatment.
Lane 7: 5 µl of the amplification product obtained with *Taq* DNA polymerase and 125 ng of *Afu* exonuclease III no subsequent UNG or heat treatment.
Lane 8: 5 µl of the amplification product obtained with *Taq* DNA polymerase and 125 ng of *Afu* exonuclease III no subsequent UNG but heat treatment.
Lane 9: DNA Molecular Weight Marker XIV (Roche Molecular Biochemicals No. 1721933)

Figure 9:

Effect of *Afu* exonuclease III on PCR product length. The *Taq* DNA polymerase/*Afu* exonuclease III mixture was analyzed on human genomic DNA as described in Example X.

Lane 1: 9,3 kb tPA fragment with Taq/Exo III Mix
Lane 2: 9,3 kb tPA fragment with Taq-Pol.
Lane 3: 12 kb tPA fragment with Taq/Exo III Mix
Lane 4: 12 kb tPA fragment with Taq-Pol.
Lane 5: 15 kb tPA fragment with Taq/Exo III Mix
Lane 6: 15 kb tPA fragment with Taq-Pol.

EXAMPLE I

Isolation of coding sequences

[0021] The preferred thermostable enzyme herein is a extremely thermostable exodeoxyribonuclease obtainable from *Archaeoglobus fulgidus* VC-16 strain (DSM No. 4304). The strain was isolated from marine hydrothermal systems at Vulcano island and Stufe di Nerone, Naples, Italy (Stetter, K. O.et al., *Science* (1987) 236:822-824).This organism is an extremely thermophilic, sulfur metabolizing, archaebacteria, with a growth range between 60°C and 95°C with optimum at 83°C. (Klenk, H.P. et al., *Nature* (1997) 390:364-370). The genome sequence is deposited in the TIGR data base. The gene putatively encoding exonuclease III (xthA) has Acc.No. AF0580.

[0022] The apparent molecular weight of the exodeoxyribonuclease obtainable from *Archaeoglobus fulgidus* is about 32,000 daltons when compared with protein standards of known molecular weight. The exact molecular weight of the thermostable enzyme of the present invention may be determined from the coding sequence of the *Archaeoglobus fulgidus* exodeoxyribonuclease III gene.

EXAMPLE II

Cloning of the gene encoding exonuclease III from *Archaeoglobus fulgidus*

[0023] About 6 ml cell culture of DSM No. 4304 were used for isolation of chromosomal DNA from *Archaeoglobus fulgidus.*
The following primers were designed with restriction sites compatible to the multiple cloning site of the desired expression vector and complementary to the N- and C-terminus of the *Archaeoglobus fulgidus* exonuclease III gene:

SEQ ID NO.: 1
N-terminus (BamHI-site): 5'-GAA ACG AGG ATC CAT GCT CAA AAT CGC CAC C -3'

SEQ ID NO.: 2
C-terminus (PstI-site): 5'-TTG TTC ACT GCA GCT ACA CGT CAA ACA CAG C -3'

First the cells were collected by repeted centrifugation in one 2 ml eppendorf cap at 5,000 rpm. The DNA isolation may be performed with any described method for isolation from bacterial cells. In this case the *Archaeoglobus fulgidus* genomic DNA was prepared with the High Pure™ PCR Template Preparation Kit (ROCHE Diagnostics GmbH, No. 1796828). With this method about 6 µg chromosomal DNA were obtained with a concentration of 72 ng/µl.

[0024] PCR was performed with the primers described above, in the Expand™ High Fidelity PCR System (ROCHE Diagnostics GmbH, No. 1732641) and 100 ng *Archaeoglobus fulgidus* genomic DNA per cap in four identical preparations. PCR was performed with the following conditions:

1 x 94°C, 2 min;
10 x 94°C, 10 sec; 54°C, 30 sec; 68°C, 3 min;
20 x 94°C, 10 sec; 54°C, 30 sec; 68°C, 3 min with 20sec cycle elongation for each cycle;
1 x 68°C, 7 min;

After adding $MgCl_2$ to a final concentration of 10 mM the PCR product was cleaved with BamHI and Pst, 10 units each, at 37°C for 2 hours. The reaction products were separated on a low-melting agarose gel. After elecrophoresis the appropriate bands were cut out, the gel slices combined, molten, the DNA fragments isolated by agarase digestion and precipitated with EtOH. The dried pellet was diluted in 30 µl H_2O.

[0025] The appropriate expression vector, here pDS56_T, was digested with the same restriction enzymes as used for the insert and cleaned with the same method.

[0026] After ligation of insert and vector with the Rapid DNA Ligation Kit (ROCHE Diagnostics GmbH, No.1635379) the plasmid was transformed in the expression host *E.coli* 392 pUBS520 (Brinkmann, U. et al. (1989) *Gene* 85:109-114).

[0027] Plasmid DNA of the transformants was isolated using the High Pure™ Plasmid Isolation Kit (ROCHE Diagnostics GmbH, No.1754777) and characterized by restriction digestion with BamHI and PstI and agarose gel electrophoresis.

[0028] Positive E.coli pUBS520 ExoIII transformants were stored in glycerol culture at -70°C. The sequence of the gene encoding exonuclease III was confirmed by DNA sequencing. It is shown in Figure No. 1.

[0029] Cloning and expression of exonuclease III from *Archaeoglobus fulgidus* or other thermophilic organisms may also be performed by other techniques using conventional skill in the art (see for example Sambrook et al. Molecular Cloning, A Laboratory Manual, Cold Spring Harbour Lab., 1989).

EXAMPLE III

Expression of recombinant Afu exonuclease III

[0030] The transformant from example I was cultivated in a fermentor in a rich medium containing appropriate antibiotic. Cells were harvested at an optical density of [A_{540}] 5.5 by centrifugation and frozen until needed or lyzed by treatment with lysozyme to produce a crude cell extract containing the *Archaeoglobus fulgidus* exonuclease III activity.

[0031] The crude extract containing the *Archaeoglobus fulgidus* exonuclease III activity is purified by the method described in example III, or by other purification techniques such as affinity-chromatography, ion-exchange-chromatography or hydrophobic-interaction-chromatography.

EXAMPLE IV

Purification of recombinant *Afu* exonuclease III

[0032] *E.coli* pUBS520 ExoIII (DSM No. 13021) from example I was grown in a 10lfermentor in media containing tryptone (20 g/l), yeast extract (10 g/l), NaCl (5 g/l) and ampicillin (100 mg/l) at 37°C, induced with IPTG (0.3 mM) at midexponential growth phase and incubated an additional 4 hours. About 45 g of cells were harvested by centrifugation and stored at - 70°C. 2 g of cells were thawed and suspended in 4 ml buffer A (40 mM Tris/HCl, pH 7.5; 0.1 mM EDTA; 7 mM 2-mercaptoethanol; lmM Pefabloc SC). The cells were lyzed under stirring by addition of 1.2 mg lysozyme for 30 minutes at 4°C and addition of 4.56 mg sodium deoxycholate for 10 minutes at room temperature followed by 20 minutes at 0°C. The crude extract was adjusted to 750 mM KCl, heated for 15 minutes at 72°C and centrifuged for removal of denatured protein.

[0033] A heating temperature up to 90 °C is also possible without destroying (denaturation) the *Archaeoglobus fulgidus* exonuclease III. The supernatant was dialyzed against buffer B (buffer A containig 10 % glycerol) adjusted to 10 mM $MgCl_2$ and applied to a Blue Trisacryl M column (SERVA, No. 67031) with the dimension 1 x 7 cm and 5.5 ml bed volume, equilibrated with buffer B. The column was washed with 16.5 ml buffer B and the exonuclease protein was eluted with a 82 ml linear gradient of 0 to 3 M NaCl in buffer B. The column fractions were assayed for *Archaeoglobus fulgidus* exodeoxyribonuclease protein by electrophoresis on 10-15% SDS-PAGE gradient gels. The active fractions, 16.5 ml, were pooled, concentrated with Aquacide II (Calbiochem No. 17851) and dialyzed against the storage buffer C (10 mM Tris/HCl, pH 7.9; 10 mM 2-mercptoethanol; 0.1mM EDTA; 50 mM KCl; 50 % glycerol). After dialysis Thesit and Nonidet P40 were added to a final concentration of 0.5% each. This preparation was stored at - 20 °C.

[0034] The *Archaeoglobus fulgidus* exonuclease III obtained was pure to 95% as estimated by SDS gel electrophoresis. The yield was 50 mg of protein per 2.3g cellmass (wetweight).

EXAMPLE V

Thermostability of recombinant exonuclease III from Archaeoglobus fulgidus

[0035] The thermostability of the exonuclease III from *Archaeoglobus fulgidus* cloned as described in Example I was determined by analyzing the resistance to heat denaturation. After lysis as described in Example III 100 µl of the crude

extract were centrifuged at 15,000 rpm for 10 min in an Eppendorf centrifuge. The supernatant was aliquoted into five new Eppendorf caps. The caps were incubated for 10 minutes at five different temperatures, 50°C, 60°C, 70°C, 80°C and 90°C. After centrifugation as described above, aliquotes of the supernatants were analyzed by electrophoresis on 10-15 % SDS-PAGE gradient gels. As shown in Figure No. 2 the amount of *Archaeoglobus fulgidus* exonuclease III protein after incubation at 90°C was the same as that of the samples treated at lower temperatures. The was no significant loss by heat denaturation detectable. From this result it can be concluded that the half life is more than ten minutes at 90°C.

EXAMPLE VI

Activity of *Afu* exonuclease III

[0036] Exonuclease III catalyzes the stepwise removal of mononucleotides from 3'-hydroxyl termini of duplex DNA (Rogers G.S. and Weiss B. (1980) *Methods Enzymol.* 65:201-211). A limited number of nucleotides are removed during each binding event. The preferred substrate are blunt or recessed 3'-termini. The enzyme is not active on single stranded DNA, and 3'-protruding termini are more resistant to cleavage. The DNA Molecular Weight Marker VI (ROCHE Molecular Biochemicals, No.1062590) consists of BglI digested pBR328 mixed with HinfI digested pBR328. The products of the HinfI digest have 3'-recessive termini and are expected to be preferred substrates to degradation by exonuclease III, the products of BglI cleavage have 3'protruding ends with 3 bases overhangs and should be more resistant to cleavage by exonuclease III.

[0037] Serial dilutions of *Archaeoglobus fulgidus* exonuclease III from Example III were incubated for 2 hours at 72 °C with 0.5 µg DNA Molecular Weight Marker VI (ROCHE Molecular Biochemicals, No.1062590) in 25 µl of the following incubation buffer: 10 mM Tris/HCl, pH 8.0; 5 mM $MgCl_2$; 1 mM 2-mercaptoethanol; 100 mM NaCl with Paraffin overlay. 10 units of exonuclease III of *E.coli* (ROCHE Molecular Biochemicals, No.779709) was included as a control. The control reaction was performed at 37°C. After addition of 5 µl stop solution (0.2 % Agarose, 60 mM EDTA, 10 mM Tris-HCl, pH 7.8, 10 % Glycerol, 0.01 % Bromphenolblue) the mixtures were separated on a 1 % agarose gel. The result is shown in Figure 3. *Afu* exonuclease III discriminates between the 2 different types of substrate. The preferred substrate are the fragments with 3'-recessive ends (e.g. 1766 bp fragment) and the 3'-overhanging ends (e.g. 2176 bp, 1230bp, 1033 bp fragments) are more resistant to degradation. With higher amounts of protein the substrate is degraded to a similar extent as in lane 1, where the products of exonuclease III of *E.coli* were analyzed. With increasing amounts of *Afu* exonuclease protein only little DNA substrate was left (lanes 15 to 19), the retardation of the remaining fragments may be due to DNA binding proteins as impurities of the preparation.

EXAMPLE VII

Mismatched primer correction in PCR with *Afu* exonuclease III

[0038] The repair efficiency of the *Afu* exonuclease III / *Taq* polymerase mixture during PCR was tested with 3' terminally mismatched primers, the principle of the assay is shown in Figure 4. For PCR amplification sets of primers are used in which the forward primer has one or two nucleotides at the 3' end which cannot base pair with the template DNA. Excision of the mismatched primer end and amplification of the repaired primer generates a product which can subsequently be cleaved with the restriction endonuclease BsiEI, whereas the product arising from the mismatched primer is resistant to cleavage.

[0039] The primer sequences used :

1. reverse: 5' - GGT TAT CGA AAT CAG CCA CAG CG - 3'
(SEQ ID NO.: 3)

2. forward 1 (g:a mismatch): 5' - TGG ATA CGT CTG AAC TGG TCA CGG TCA - 3'
(SEQ ID NO.: 4)

3. forward 2 (g:t mismatch): 5' - TGG ATA CGT CTG AAC TGG TCA CGG TCT - 3'
(SEQ ID NO.: 5)

4. forward 3 (g:c mismatch): 5' - TGG ATA CGT CTG AAC TGG TCA CGG TCC - 3'
(SEQ ID NO.: 6)

5. forward 4 (2 base mismatch): 5' - TGG ATA CGT CTG AAC TGG TCA CGG TAT - 3'
(SEQ ID NO.: 7)

PCR was carried out using 2.5 Units *Taq* DNA Polymerase (ROCHE Diagnostics GmbH, No. 1435094), 0.25 µg of *Archaeoglobus fulgidus* exonuclease III from Example III, 10 ng of DNA from bacteriophage λ, 0.4 µM of each primer, 200 µM of dNTP's, 1.5 mM of $MgCl_2$, 50 mM of Tris-HCl, pH 9.2, 16 mM of $(NH_4)_2SO_4$. PCR was performed in an volume of 50µl PCR with the following conditions:

1 x 94°C, 2 min;
40 x 94°C, 10 sec; 60°C, 30 sec; 72°C, 1 min;
1 x 72°C, 7 min;

The function of the exonuclease/Taq polymerase mixture was compared to controls as 2.5 Units of *Taq* DNA polymerase, 0.3 Units of *Tgo* DNA polymerase (ROCHE Diagnostics GmbH) and to 0.75 µl of Expand™ High Fidelity PCR System (ROCHE Diagnostics GmbH, No.1732641). As indicated by successfull digestion of the PCR products with BsiEI *A. fulgidus* exonuclease III showed correcting activity of all described mismatches with an effectivity of 90 to 100 % (Figure 5). *Taq* DNA Polymerase as expected showed no correcting activity, while *Tgo* DNA Polymerase with it's 3'-5'exonuclease activity corrected completely as well. The Expand™ High Fidelity PCR System showed only with the two base mismatch 100% correcting activity. The other mismatches were repaired with an effectivity of approximately 50%.

EXAMPLE VIII

Fidelity of *Afu* exonuclease *III/Taq* DNA polymerase mixtures in the PCR process

[0040] The fidelity of Afu exonuclease *III/Taq* DNA polymerase mixtures in the PCR process was determined in an assay based on the amplification, circularisation and transformation of the pUC19 derivate pUCIQ17, containing a functional *lac* I^q allele (Frey, B. and Suppmann B. (1995) *Biochemica* 2:34-35). PCR-derived mutations in *lac* I are resulting in a derepression of the expression *of lac Z*α and subsequent formation of a functional β-galactosidase enzyme which can be easily detected on X-Gal indicator plates . The error rates of *Taq* polymerase/*Afu* exonuclease mixtures determined with this *lac* I-based PCR fidelity assay were determined in comparison to *Taq* DNA polymerase and Expand HiFi PCR System (Roche Molecular Biochemicals) and *Pwo* DNA polymerase (Roche Molecular Biochemicals) as controls.

[0041] The plasmid pUCIQ17 was linearized by digestion with DraII to serve as a substrate for PCR amplification with the enzymes tested.

[0042] Both of the primers used have ClaI sites at their 5 prime ends:

SEQ ID NO.: 8

Primer 1: 5'-AGCTTATCGATGGCACTTTTCGGGGAAATGTGCG-3'

SEQ ID NO.: 9

Primer 2: 5'-AGCTTATCGATAAGCGGATGCCGGGAGCAGACAAGC-3'

The length of the resulting PCR product is 3493 pb.
The PCR was performed in a final volume of 50 µl in the presence of 1.5 mM $MgCl_2$, 50 mM Tris· HCl, pH 8.5 (25°C), 12.5 mM $(NH4)_2SO_4$, 35 mM KCl, 200 µM dNTPs and 2.5 units of *Taq* polymerase and 125 ng, 175 ng, 250 ng, 375 ng and 500 ng, respectively of *Afu* exonuclease III.

[0043] The cycle conditions were as follows:

1 x denaturation of template for 2 min. at 95°C

8 x
- denaturation at 95°C for 10 sec.
- annealing at 57°C for 30 sec.
- elongation at 72°C for 4 min.

16 x
- denaturation at 95°C for 10 sec.
- annealing at 57°C for 30 sec.
- elongation at 72°C for 4 min.
- + cycle elongation of 20 sec. for each cycle

After PCR, the PCR products were PEG-precipitated (Barnes, W. M. (1992) *Gene* 112:229) the DNA restricted with ClaI and purified by agarose gel electrophoresis. The isolated DNA was ligated using the Rapid DNA Ligation Kit (Roche Molecular Biochemicals) and the ligation products transformed in *E.coli* DH5α, plated on TN Amp X-Gal plates. The α-complementing *E.coli* strain DH5α transformed with the resulting plasmid pUCIQ17 (3632 bp), shows white (lacI+) colonies on TN plates (1.5 % Bacto Tryptone, 1 % NaCl, 1.5 % Agar) containing ampicillin (100 µg/ml) and X-Gal (0.004 % w/v). Mutations result in blue colonies.

[0044] After incubation overnight at 37°C, blue and white colonies were counted. The error rate (f) per bp was calculated with a rearranged equation as published by Keohavong and Thilly (Keohavong, P. and Thilly, W. (1989) *PNAS* USA 86:9253):

$$f=-\ln F/d \times b\ bp$$

where F is the fraction of white colonies:

$$F = \text{white (lacI+) colonies / total colony number;}$$

d is the number of DNA duplications:

$$2^d = \text{output DNA / input DNA;}$$

and **b** is the effective target size of the (1080bp) *lac* I gene, which is 349 bp according to Provost et al. (Provost et al. (1993) *Mut. Res.* 288:133).

[0045] The results shown in Figure 6A and Figure 6B demonstrate that the presence of thermostable exonuclease III in the reaction mixure results in lower error rates. Dependent on the ratio of polymerase to exonuclease the error rate is decreasing. The fidelity achieved with the most optimal *Taq* polymerase / *Afu* exonuclease III mixture (4,44 x

10^{-6}) is in a similar range as that of the *Taq/Pwo* mixture (Expand HiFi; 2,06 x 10^{-6}). Evaluation of the optimal buffer conditions will further improve the fidelity. The ratio between polymerase and exonuclease has to be optimized. High amounts of exonuclease reduce product yield, apparently decreasing amplification efficiency (Taq/Exo 5 corresponding to 2.5 units of *Taq* polymerase and 500 ng of *Afu* exonuclease III).

[0046] The fidelity of this system may further be optimized using conventional skill in the art e.g. by altering the buffer components, optimizing the concentration of the individual components or changing the cycle conditions.

EXAMPLE IX:

Incorporation of dUTP in the presence of *Afu* exonuclease III during PCR

[0047] The *Afu* exonuclease /*Taq* polymerase mixture was tested for DNA synthesis with TTP completely replaced by dUTP. Comparisation of either TTP or UTP incorporation was determinated in PCR using 2.5 Units of *Taq* DNA Polymerase, in presence of 0.125 µg, 0.25 µg, 0.375 µg and 0.5 µg of *Archaeoglobus fulgidus* exonuclease III from example III on native human genomic DNA as template using the β-globin gene as target. The following primers were used:

forward:	5' - TGG TTG AAT TCA TAT ATC TTA GAG GGA GGG C – 3' (SEQ ID NO.: 10)
reverse:	5' - TGT GTC TGC AGA AAA CAT CAA GGG TCC CAT A - 3' (SEQ ID NO.: 11)

PCR was performed in 50 µl volume with the following cycle conditions:

1 x 94°C, 2 min;
40 x 94°C, 10 sec; 60°C, 30 sec; 72°C, 1 min;
1 x 72°C, 7 min;

Aliquots of the PCR reaction were separated on agarose gels. As shown in Figure 7 DNA synthesis in the presence of dUTP is possible with up to 375 ng of Afu exonuclease III. dUTP incorporation can further be proven by Uracil-DNA Glycosylase treatment (ROCHE Diagnostics GmbH, No.1775367) of aliquotes from the PCR reaction products for 30 min at ambient temperature and subsequent incubation for 5 min at 95°C to cleave the polynucleotides at the apurinic sites which leads to complete degradation of the fragments. The analysis of the reaction products by agarose gel electrophoresis is shown in Figure 8.

EXAMPLE X:

Effect of Afu exonuclease III on PCR product length

[0048] *Taq* polymerase is able to synthesize PCR products up to 3 kb in length on genomic templates. In order to estimate the capability of the *Taq* polymerase/*Afu* exonuclease mixture for the synthesis of longer products, the enzyme mixture was analyzed on human genomic DNA as template with three pairs of primers designed to amplify products of 9.3 kb, 12 kb and 15 kb lenght. The buffer systems used were from the Expand Long Template PCR System (Roche Molecular Biochemicals Cat. No 1 681 834). Reactions were performed in 50 µl volume with 250 ng of human genomic DNA, 220 ng of each primer, 350 µM of dNTPs and 2.5 units of *Taq* polymerase and 62,5 ng of *Afu* exonuclease with the following conditions:

Product length	Primers	Expand Long Template buffer No.:	PCR Programm
9.3 kb	forward 7 reverse 14	1	1 x denat. at 94 °C for 2 min
			10 x denat. at 94°C for 10 sec. annealing at 65°C for 30 sec elogation at 68°C for 8 min.
			20 x denat. at 94°C for 10 sec. annealing at 65°C for 30 sec elogation at 68°C for 8 min. plus cycle elongation of 20 sec. per cycle
			1 x elongation at 68°C for 7 min.
12 kb	forward 1 reverse 3	2	1 x denat. at 94 °C for 2 min
			10 x denat. at 94°C for 10 sec. annealing at 62°C for 30 sec elogation at 68°C for 12 min.
			20 x denat. at 94°C for 10 sec. annealing at 62°C for 30 sec elogation at 68°C for 12 min. plus cycle elongation of 20 sec. per cycle
			1 x elongation at 68°C for 7 min.
15 kb	forward 1 reverse 2	3	same as for 12 kb

[0049] The primer specific for amplification of the tPA genes used:

Primer 7 forward: 5' - GGA AGT ACA GCT CAG AGT TCT GCA GCA CCC CTG C - 3'
(SEQ ID NO.: 12)

Primer 14 reverse: 5 '- CAA AGT CAT GCG GCC ATC GTT CAG ACA CAC C - 3'
(SEQ ID NO.: 13)

Primer 1 forward: 5 '- CCT TCA CTG TCT GCC TAA CTC CTT CGT GTG TCC C- 3'
(SEQ ID NO.: 14)

Primer 2 reverse: 5' - ACT GTG CTT CCT GAC CCA TGG CAG AAG CGC CTT C- 3'
(SEQ ID NO.: 15)

Primer 3 reverse: 5 '- CCT TCT AGA GTC AAC TCT AGA TGT GGA CTT AGA G - 3'
(SEQ ID NO.: 16)

As shown in Figure 9 it is possible to synthesize products of at least 15 kb in length with the *Taq* polymerase/*Afu* exonuclease mixture.

Claims

1. Thermostable enzyme exhibiting 3' exonuclease-activity but no polymerase activity whereas this enzyme enhances fidelity of an amplification process when added to a second enzyme exhibiting polymerase activity.

2. Thermostable enzyme according to claim 1 obtainable from *Archeoglobus fulgidus.*

3. Thermostable enzyme according to claim 1 or 2 whereas this enzyme is able to cooperate as proofreading enzyme with a second enzyme exhibiting polymerase activity.

4. Composition comprising a first thermostable enzyme exhibiting 3'-exonuclease-activity but no polymerase activity and a second enzyme exhibiting polymerase activity whereas the fidelity of an amplification process is enhanced by the use of this composition in comparison to the use of the second enzyme alone.

5. Composition according to claim 4 whereas the second enzyme is lacking proofreading activity.

6. Composition according to claim 4 whereas the second enzyme is Taq polymerase.

7. A method of DNA synthesis using a mixture according to claim 5 or 6.

8. A method of claim 7 whereas prematurely terminated chains are trimmed by degradation from 3' to 5'.

9. A method according to one of the claims 7-8 whereas mismatched ends of either a primer or the growing strand are removed

10. A method according to one of the claims 7-10 whereas dUTP is present in the reaction mixture, replacing TTP.

11. A method according to claim 10 whereas UNG is used for degradation of contaminating nucleic acids.

12. A method according to one of the claims 7-11 whereas the mixture of a

- first thermostable enzyme exhibiting 3'-exonuclease-activity but no polymerase activity and
- a second enzyme exhibiting polymerase activity

produces PCR products with lower error rates compared to PCR products produced by the second enzyme exhibiting polymerase activity in absence of the first thermostable enzyme exhibiting 3'-exonuclease-activity but no polymerase activity.

13. A method of claim 12 in which the mixture of first thermostable enzyme exhibiting 3'-exonuclease-activity but no polymerase activity and a second enzyme exhibiting polymerase activity produces PCR products of greater length compared to PCR products produced by the second enzyme exhibiting polymerase activity in absence of the first thermostable enzyme exhibiting 3'-exonuclease-activity but no polymerase activity

14. A method according to one of the claims 7-14 whereas the first thermostable enzyme exhibiting 3'-exonuclease-activity but no polymerase activity is related to the Exonuclease III of E.coli, but thermostable.

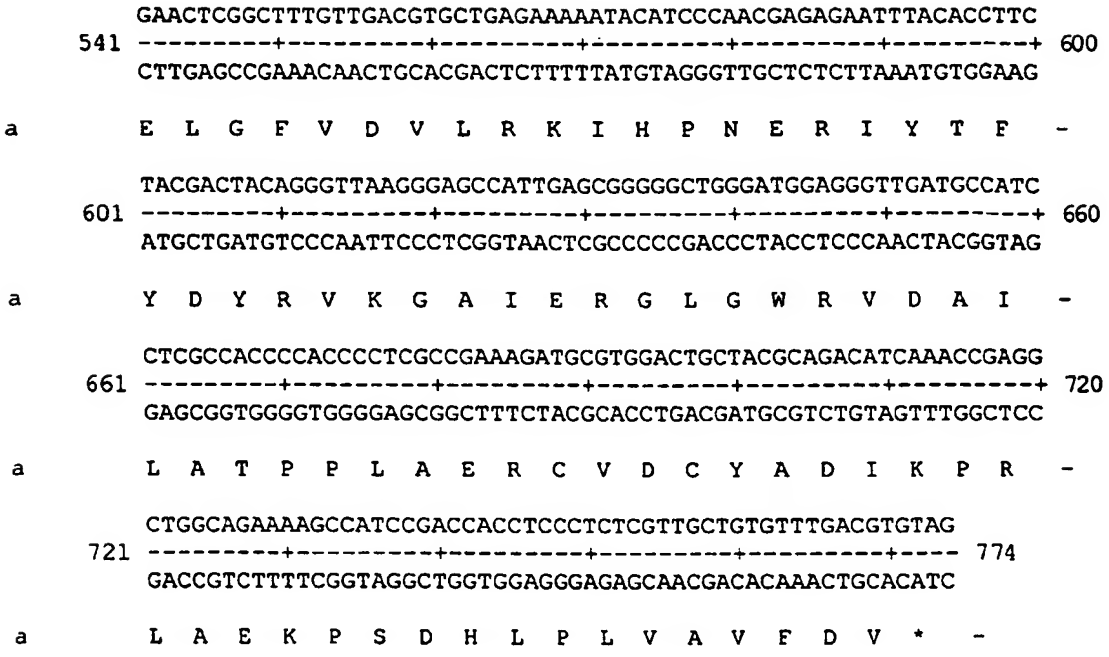
15. A method according to one of the claims 7-14 whereas PCR products with blunt ends are obtained.

Figure 1: 1/2

Sequence of the *Archaeoglobus fulgidus* exonuclease III gene:
SEQ ID NO.: 17

```
          ATGCTCAAAATCGCCACCTTCAACGTAAACTCCATCAGGAGCAGACTGCACATCGTGATT
        1 ---------+---------+---------+---------+---------+---------+ 60
          TACGAGTTTTAGCGGTGGAAGTTGCATTTGAGGTAGTCCTCGTCTGACGTGTAGCACTAA

a           M  L  K  I  A  T  F  N  V  N  S  I  R  S  R  L  H  I  V  I  -

          CCGTGGCTGAAGGAGAACAAGCCTGACATTCTATGCATGCAGGAGACGAAGGTTGAGAAC
       61 ---------+---------+---------+---------+---------+---------+ 120
          GGCACCGACTTCCTCTTGTTCGGACTGTAAGATACGTACGTCCTCTGCTTCCAACTCTTG

a           P  W  L  K  E  N  K  P  D  I  L  C  M  Q  E  T  K  V  E  N  -

          AGGAAGTTTCCTGAGGCCGATTTTCACCGCATCGGCTACCACGTCGTCTTCAGCGGGAGC
      121 ---------+---------+---------+---------+---------+---------+ 180
          TCCTTCAAAGGACTCCGGCTAAAAGTGGCGTAGCCGATGGTGCAGCAGAAGTCGCCCTCG

a           R  K  F  P  E  A  D  F  H  R  I  G  Y  H  V  V  F  S  G  S  -

          AAGGGAAGGAATGGAGTGGCCATAGCTTCCCTCGAAGAGCCTGAGGATGTCAGCTTCGGT
      181 ---------+---------+---------+---------+---------+---------+ 240
          TTCCCTTCCTTACCTCACCGGTATCGAAGGGAGCTTCTCGGACTCCTACAGTCGAAGCCA

a           K  G  R  N  G  V  A  I  A  S  L  E  E  P  E  D  V  S  F  G  -

          CTCGATTCAGAGCCGAAGGACGAGGACAGGCTGATAAGGGCAAAGATAGCTGGCATAGAC
      241 ---------+---------+---------+---------+---------+---------+ 300
          GAGCTAAGTCTCGGCTTCCTGCTCCTGTCCGACTATTCCCGTTTCTATCGACCGTATCTG

a           L  D  S  E  P  K  D  E  D  R  L  I  R  A  K  I  A  G  I  D  -

          GTGATTAACACCTACGTTCCTCAGGGATTCAAAATTGACAGCGAGAAGTACCAGTACAAG
      301 ---------+---------+---------+---------+---------+---------+ 360
          CACTAATTGTGGATGCAAGGAGTCCCTAAGTTTTAACTGTCGCTCTTCATGGTCATGTTC

a           V  I  N  T  Y  V  P  Q  G  F  K  I  D  S  E  K  Y  Q  Y  K  -

          CTCCAGTGGCTTGAGAGGCTTTACCATTACCTTCAAAAAACCGTTGACTTCAGAAGCTTT
      361 ---------+---------+---------+---------+---------+---------+ 420
          GAGGTCACCGAACTCTCCGAAATGGTAATGGAAGTTTTTTGGCAACTGAAGTCTTCGAAA

a           L  Q  W  L  E  R  L  Y  H  Y  L  Q  K  T  V  D  F  R  S  F  -

          GCTGTTTGGTGTGGAGACATGAACGTTGCTCCTGAGCCAATCGACGTTCACTCCCCAGAC
      421 ---------+---------+---------+---------+---------+---------+ 480
          CGACAAACCACACCTCTGTACTTGCAACGAGGACTCGGTTAGCTGCAAGTGAGGGGTCTG

a           A  V  W  C  G  D  M  N  V  A  P  E  P  I  D  V  H  S  P  D  -

          AAGCTGAAGAACCACGTCTGCTTCCACGAGGATGCGAGAAGGGCATACAAAAAAATACTC
      481 ---------+---------+---------+---------+---------+---------+ 540
          TTCGACTTCTTGGTGCAGACGAAGGTGCTCCTACGCTCTTCCCGTATGTTTTTTTATGAG

a           K  L  K  N  H  V  C  F  H  E  D  A  R  R  A  Y  K  K  I  L  -
```

Figure 1: 2/2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541 ---------+---------+---------+---------+---------+---------+ 600
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a E L G F V D V L R K I H P N E R I Y T F -
TACGACTACAGGGTTAAGGGAGCCATTGAGCGGGGGCTGGGATGGAGGGTTGATGCCATC
601 ---------+---------+---------+---------+---------+---------+ 660
ATGCTGATGTCCCAATTCCCTCGGTAACTCGCCCCCGACCCTACCTCCCAACTACGGTAG
a Y D Y R V K G A I E R G L G W R V D A I -
CTCGCCACCCCACCCCTCGCCGAAAGATGCGTGGACTGCTACGCAGACATCAAACCGAGG
661 ---------+---------+---------+---------+---------+---------+ 720
GAGCGGTGGGGTGGGGAGCGGCTTTCTACGCACCTGACGATGCGTCTGTAGTTTGGCTCC
a L A T P P L A E R C V D C Y A D I K P R -
CTGGCAGAAAAGCCATCCGACCACCTCCCTCTCGTTGCTGTGTTTGACGTGTAG
721 ---------+---------+---------+---------+---------+---- 774
GACCGTCTTTTCGGTAGGCTGGTGGAGGGAGAGCAACGACACAAACTGCACATC
a L A E K P S D H L P L V A V F D V * -

Figure 2:

Temperature stability of *Afu* exonuclease III

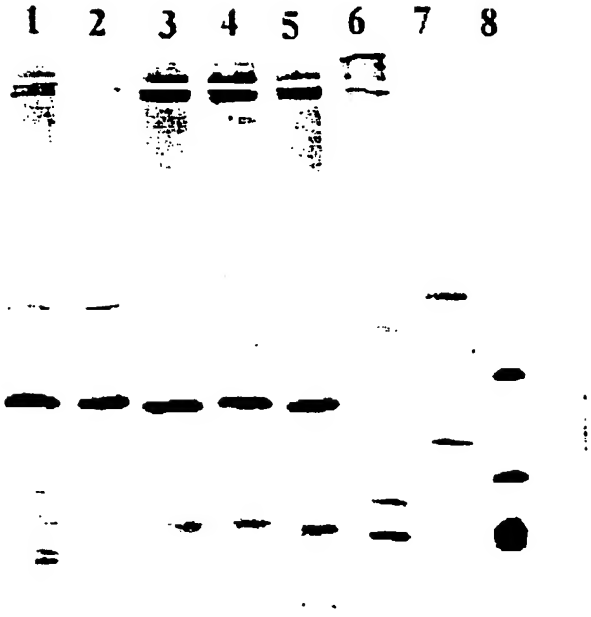


Figure 3:

Test for exonuclease III activity

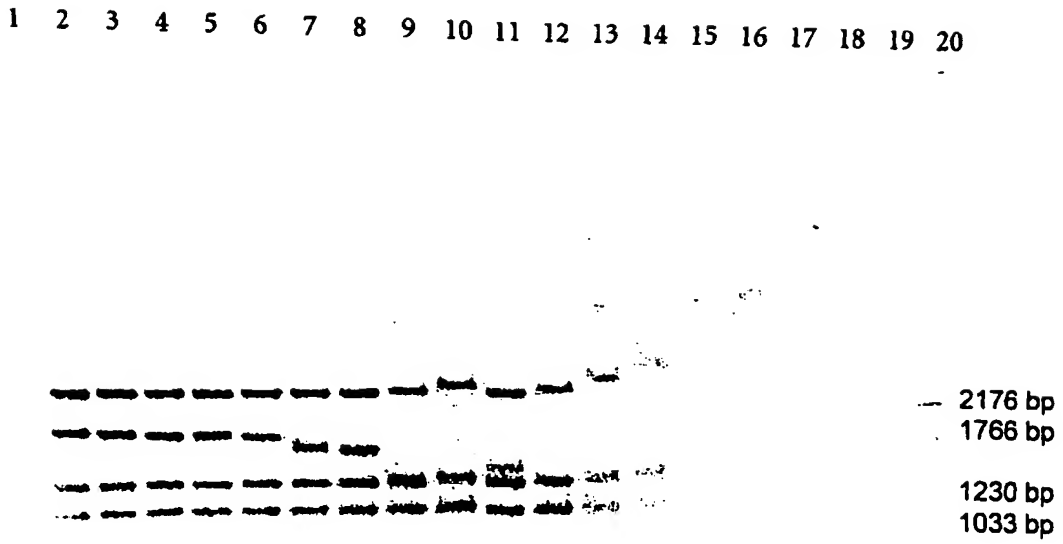


Figure 4:

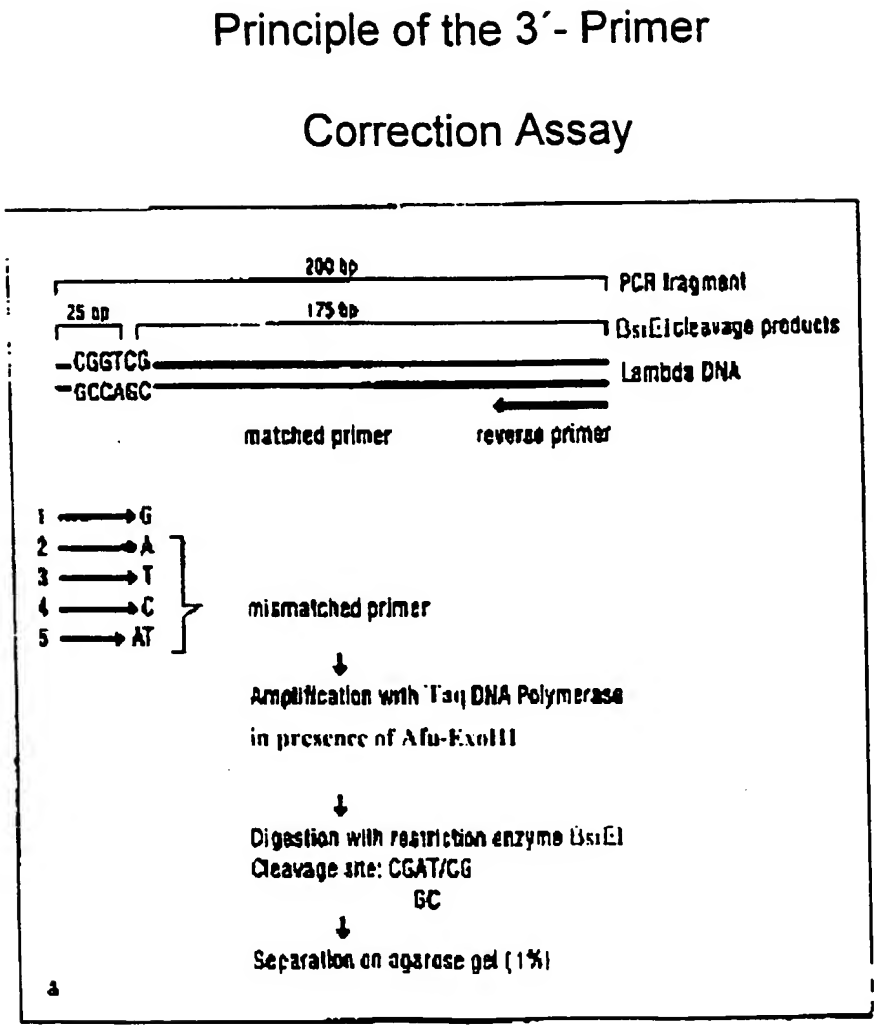

Principle of the 3´- Primer
Correction Assay
200 bp
PCR fragment
25 bp
175 bp
BsiEI cleavage products
CGGTCG
GCCAGC
Lambda DNA
matched primer
reverse primer
1 → G
2 → A
3 → T
4 → C
5 → AT
mismatched primer
Amplification with Taq DNA Polymerase
in presence of Afu-ExoIII
Digestion with restriction enzyme BsiEI
Cleavage site: CGAT/CG
GC
Separation on agarose gel (1%)
a

Figure 5:

Mismatched primer correction in PCR

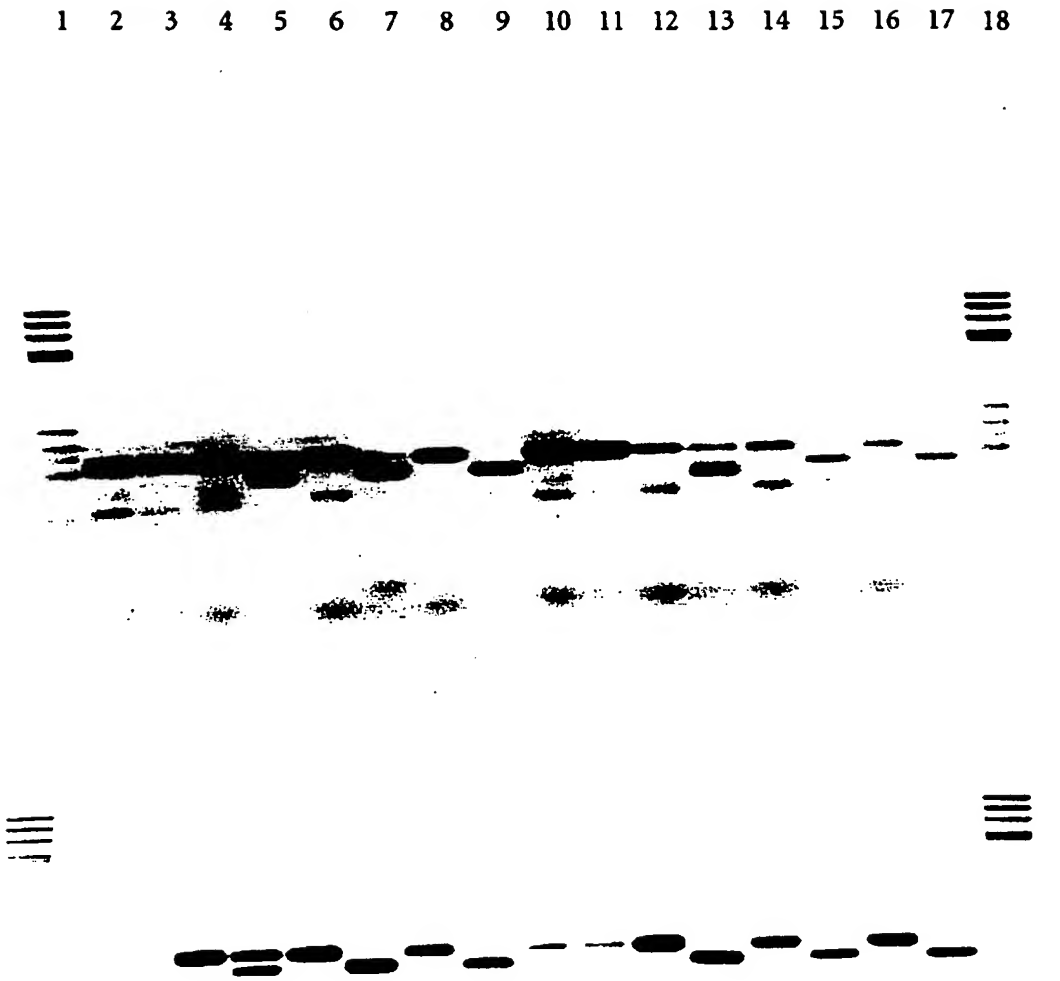


Figure 6A

Error rates of different DNA polymerases in PCR

Polymerase	Template conc. (ng)	yield (ng)	DNA duplica-tions d	blue colonies lacI⁻	white colonies lac⁺	total number of colonies	% lac⁻	error rate (f_{349})
Taq Ch.	10	11650	10.2	130	2261	2391	5.4	1.57×10^{-5}
HiFi Ch.	10	11550	10.2	40	5458	5498	0.72	2.06×10^{-6}
Pwo	10	9675	9.9	17	5891	5908	0.29	8.32×10^{-7}
Taq/Exo 1	10	11550	10.2	94	4291	4385	2.14	6.10×10^{-6}
Taq/Exo 2	10	11125	10.1	146	7644	7790	1.87	5.36×10^{-6}
Taq/Exo 3	10	8500	9.7	133	8188	8321	1.6	4.74×10^{-6}
Taq/Exo 4	10	1292	7	79	7236	7315	1.08	4.44×10^{-6}
Taq/Exo 5	10	236	4.6	25	2674	2724	0.92	1.16×10^{-5} (*)

* Due to the unfavorable ratio of Taq:Exo the product yield was low. This results in an apparently low amplification efficiency d, which is an important parameter in the formula used for the calculation of the error rate.

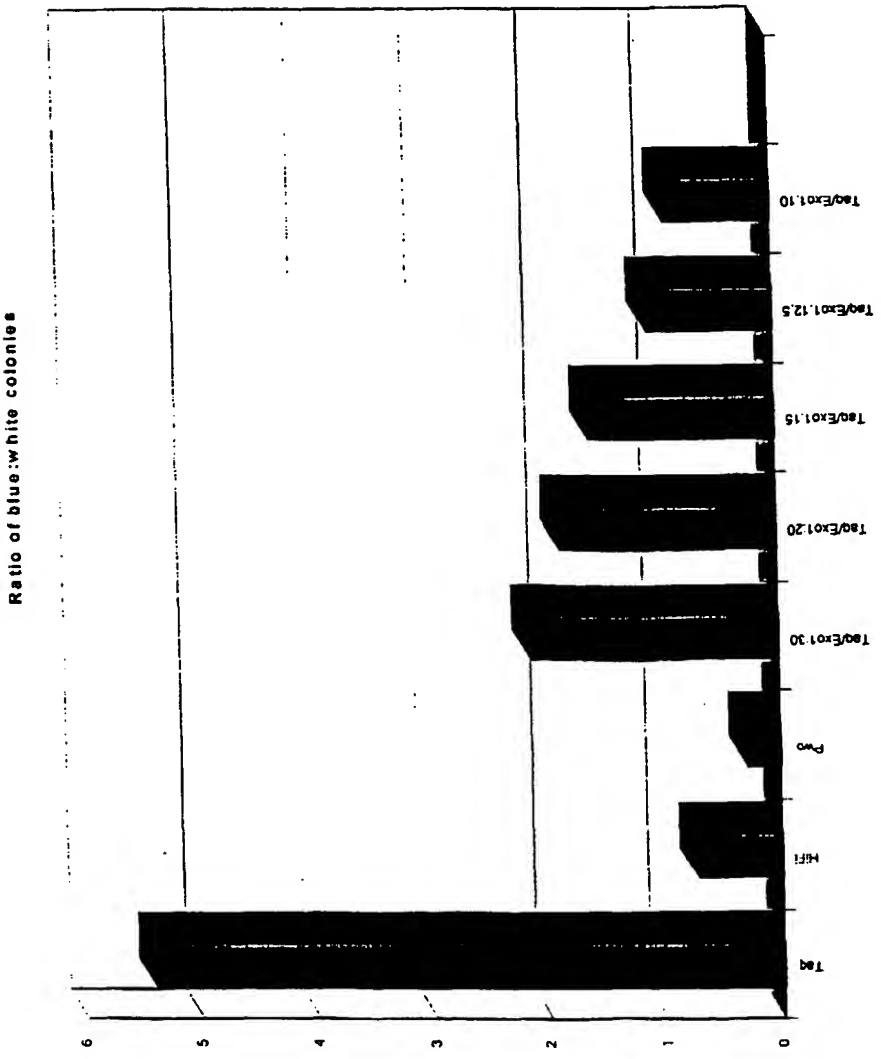

Ratio of blue:white colonies
0
1
2
3
4
5
6
Taq
HiFi
Pwo
Taq/Exo1.30
Taq/Exo1.20
Taq/Exo1.15
Taq/Exo1.12.5
Taq/Exo1.10

FIGURE 6B

Figure 7:

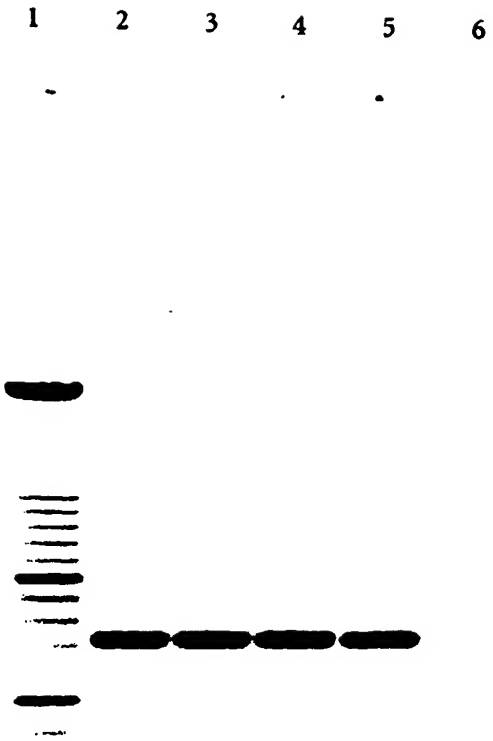


Figure 8:

UNG treatment of dUMP containing PCR products

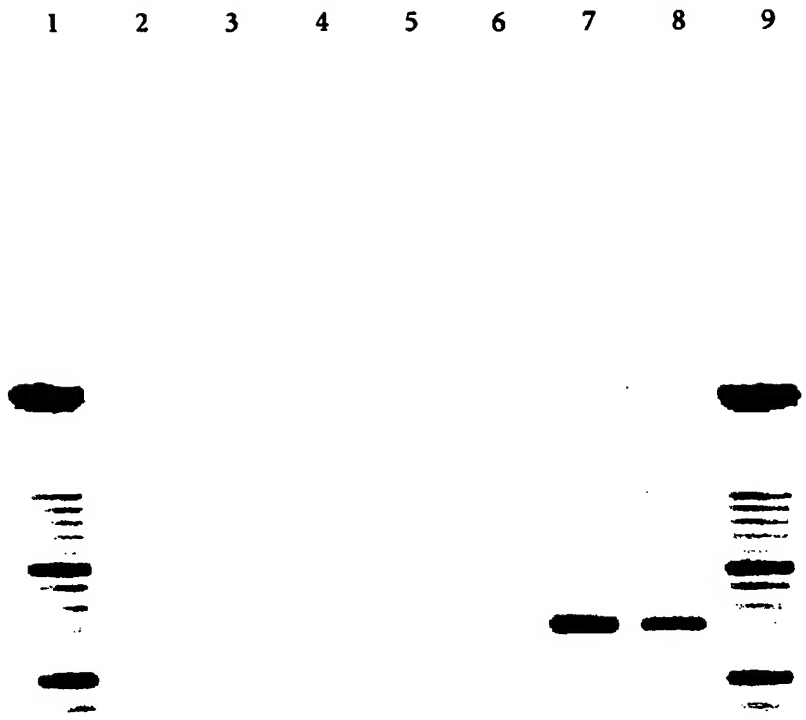
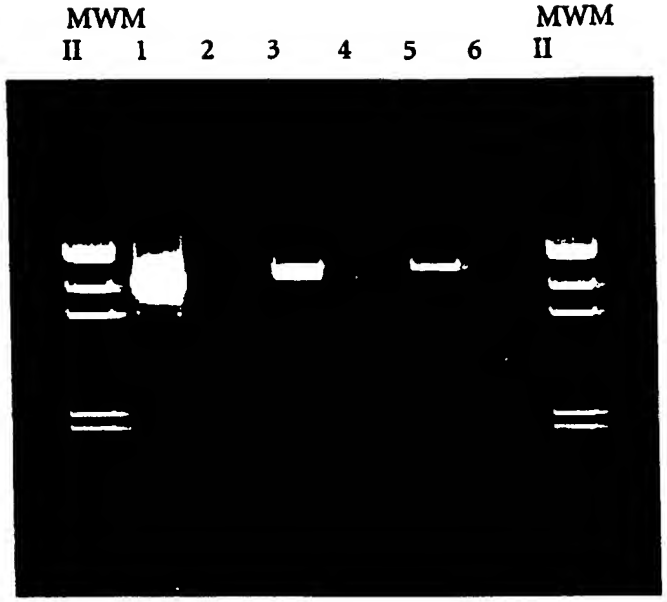


Figure 9:



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EUROPEAN SEARCH REPORT

Application Number
EP 99 11 9268

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The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
THE HAGUE	31 May 2000	Hornig, H

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